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13. ABSTRACT (Maximum 200 Words)  Mutations in the AR, changes in growth factor signaling pattern or amplification of the AR may be responsible for androgen independent prostate cancer (AIPC). The aim of this project was to look for changes in the AR in the tumors of patients with AIPC. Due to poor preservation of DNA and low frequency of the AR mutations in available samples I studied two different set of samples. Tumors from patients with prostate cancer before and after androgen ablation therapy (AA) and lymph node metastases from patients who did not receive any AA therapy. In this report I describe the identification of three AR mutants. S863P isolated from lymph node metastases does not bind R1881 and is transcriptionally inactive regardless of the ligands tested. K580R, another lymph node metastatic, DNA binding domain mutant, shows promoter and cell type specific transcriptional activity. Of the 10 patients analyzed before and after AA therapy, one patient showed an expansion of poly-glutamine repeat (from Q20-Q26) following AA therapy. ARQ26 shows reduced transcriptional activity compared to the ARQ20. Future work will include further characterization of the identified mutants and screening of tumors with and without the AR mutations for the activation of MAPK.				
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**Introduction:**

Prostate cancer is a major health problem in the western world and is the second leading cause of cancer death in men in the United States, exceeded only by lung cancer. The American Cancer Society estimates that 31,500 men in the United States will die of prostate cancer during 2001. Prostate cancer accounts for about 11% of male cancer-related deaths. It is therefore an active area of research.

Circulating androgens and a transcriptionally active androgen receptor (AR) are required for growth and maintenance of both the prostate and prostate cancer. In the case of prostate cancer, androgen ablation therapy results in regression of tumor initially, but in the majority of cases the tumor progresses from androgen dependent to an androgen independent state within a short time. In androgen independent tumors, the malignant cells continue to express functional AR. The main focus of this project was to develop an understanding of the mechanism by which the tumors acquire androgen independence. Several androgen receptor dependent mechanisms may explain androgen independence. These include 1. Mutations in the AR that allow the receptor to respond to a wider range of hormones. 2. Amplification of the AR so that a low level of hormone is sufficient to provide enough active AR. 3. Changes in growth factor signaling that activates the receptor in an environment of little or no androgen. The aim of my project was to look at metastatic tumors of prostate cancer in patients that have failed androgen ablation therapy. The AR in these tumors is presumably being activated by one of the mechanisms described above.

**Final report:**

The statement of works required the analysis of tumors from multiple sites in patients who failed androgen ablation therapy. One, to look for changes in androgen receptor and perform functional analysis of the mutations identified in the samples and two, to look for changes in receptor function. Although the period for this fellowship has ended, I will continue to work on this and related projects for atleast another year with Dr Weigel. This period will enable me to finish the experiments and submit the results for atleast two publications. Therefore, the experiments proposed in the

statement of works that are not included in this final report will be done by the completion of the project.

As outlined in the annual report 2000, we had difficulties in obtaining samples with sufficient preservation of DNA. In the absence of those samples, two sets of tumors were studied. Metastatic tumors isolated from patients that have not undergone any androgen ablation therapy and a set of tumors obtained before and after androgen ablation therapy. To this end I have looked at quite a few samples in collaboration with Dr Dolores Lamb and Dr Marco Marcelli and have characterized three different mutants.

The detection of mutations was to be performed by single strand conformation polymorphism (SSCP) and any aberrant pattern was to be analyzed by direct sequencing for the identification of mutations. However, with the availability of the automated DNA sequencer that can analyze large number of samples, the need to look for SSCP was considered superfluous. The samples were therefore analyzed by direct sequencing only.

I did perform analysis of some of the tumors, which meant isolation of DNA from the tumor samples, amplification of the AR by PCR and then sequencing of the amplified product. But I along with my post-doctoral advisor, Dr Weigel felt that repetition of the same technique (identification of mutations in the tumor samples) does not have any training value and therefore a technician in the lab of Dr Lamb performed the analysis on the majority of samples. Following the identification of mutations, functional characterization of the mutant receptors was performed by me.

The following is a brief description of some of the AR mutants studied. Characterization of two of these (Expanded poly-glutamine repeat, S863P) mutants is complete. The rest are still under investigation.

**1. Expansion of poly-glutamine repeats following androgen ablation therapy:** In a screening of 10 patients, performed in Dr Marcelli's lab, cancer specimens were obtained before and after androgen ablation therapy. One patient was found to have an expansion of poly-glutamine repeats from 20 (100% of the specimen) to 26 (70% of the specimen). Initial analysis performed in Dr Marcelli's lab showed that AR Q26 translocates to the nucleus

and binds  $^3\text{H}$ -DHT with affinity equal to that of ARQ20. My results show that ARQ26 is transcriptionally less active than ARQ20. A manuscript outlining these results has been submitted to the Journal of Clinical Endocrinology and Metabolism.

2. **AR mutants in lymph node metastases:** In a screening performed in Dr Lamb's lab, metastatic tumors were isolated from patients that have not undergone any androgen ablation therapy. A hormone binding domain mutant (S863P) and a DNA binding domain mutant (K580R) were isolated from this screening. The results of the functional analysis of these mutants are described below.

**S863P:** In this mutant, the serine residue at position 863 is changed to proline. I have found that this mutant does not bind R1881 and is transcriptionally inactive regardless of the ligands tested, these include R1881, testosterone, dihydrotestosterone, and estradiol. This mutant is also unable to be activated in a ligand independent manner.

**K580R:** This is a DNA binding domain mutant where lysine at position 580 is changed to arginine. Experiments to test the transcriptional activity of this mutant show that the transcriptional activity of mutant AR is both cell line and promoter dependent. In COS-1 cells, the transcriptional activity of K580R is lower compared to wild type when the simple GRE consensus sequence as well as the MMTV promoter was used. Both these promoters have chloramphenicol acetyl transferase (CAT) reporter gene. Interestingly however, when K580R was tested for transcriptional activity using the GRE consensus sequence in front of a luciferase reporter gene, no transcriptional activity was observed. The difference in these two promoters is the presence of an AP-1 binding site in the GRE-CAT reporter. A GRE-CAT reporter plasmid devoid of the AP-1 binding site has been generated in the lab and this mutant will be tested for transcriptional activity using this reporter plasmid. In addition, K580R does not activate transcriptional activity from the probasin promoter in COS-1 cells. In PC-3 cells on the other hand, the probasin promoter is activated at levels comparable to the MMTV or GRE promoter. In addition to the promoters already mentioned, transcriptional activity of this mutant using the C3 enhancer region and sex-limited protein promoter (slp) is also being determined in both cell types.



DNA binding specificity of AR is under intense investigation by many laboratories. Work from other laboratories has shown that residues T585, G610 and L617 in the second zinc finger of AR DNA binding domain are important in binding to the probasin promoter. Our observation that the K580 residue is also important in interaction with probasin promoter but not the GRE consensus sequence and that this interaction is cell type specific is very interesting.

**Inhibition of transcriptional activity by the AR:** In addition to activating transcription through binding to DNA, the AR interacts with other transcription factors and modulates their activity. The proteins belonging to the family of NFkB is among them. I have established assays for measuring the effect of the AR on Rel A dependent transcription as well as of Rel A on the AR dependent transcription. It will be interesting to study the effect of mutations in the AR on the transcription of genes involved in cell proliferation.

As described in my last annual report, I had changed my research plan slightly. I had proposed to take a subset of samples from patients that have failed androgen ablation therapy. This set of samples was to consist of those AR that have been identified as having mutations, as well as some that have no mutations. I had proposed to determine, as originally planned, whether the AR is amplified or the MAPK is activated. In several patients, Dr Lamb has detected mutations identical to the LNCaP mutation. These samples were to be included in my study. I had predicted that the altered hormone binding of the LNCaP AR mutant is sufficient to induce androgen independence and that I will see the AR gene amplification and/or MAPK activation more frequently in tumors that do not exhibit this mutation.

With regard to the amplification of AR, a study published earlier this year by Marika et al., (2001) showed a 6-fold increase in AR expression in hormone refractory tumors compared to androgen-dependent tumors. These tumors also showed AR gene amplification, determined by *in situ* hybridization. With the publication of this study, I, under the advice of Dr Weigel abandoned the experiments designed to look for AR amplification in prostate cancer patients.

With regard to the experiments designed to determine the MAPK activation, unfortunately we are still trying to get samples to perform those experiments. The acquisition of the samples has partly been hampered by the state of emergency at the Texas Medical Center. With the things slowly coming back to normal, I am hopeful that I will be able to complete the work with the next few months.

**Key accomplishments:**

- Learned techniques used in processing of tumor samples such as isolation of DNA, PCR amplification of the AR, sequencing of the AR.
- Isolation of the AR mutants from patients with metastatic disease.
- Partial characterization of the isolated mutants.

**Reportable outcomes:**

A manuscript reporting part of the work described in the report has been submitted to the Journal of Clinical Endocrinology and Metabolism.

Part of the work presented in this report was presented at the annual Endocrine Society meeting in June 2000. The abstract is enclosed.

I was awarded a travel award by Women in Endocrinology to present part of this work at the annual Endocrine Society meeting in June 2000.

**Conclusions:**

In summary, we have performed a study on patients with prostate cancer. The study included two sets of patients, untreated and androgen ablation treated patients. We identified three mutants. A DNA binding domain mutant, a ligand binding domain mutant and a mutant with expanded glutamine repeats. It is interesting to note that two of the mutants identified have a lower transcriptional activity than the wild type AR, while the third mutant was inactive. Lower activity AR or inactive AR mutants have also been isolated in similar studies performed in the lab. This is an important observation since androgen receptor activity is required the maintenance of prostate cancer. The possible explanation for this observation can be that the prostate cancer cells are undergoing dedifferentiation and lower androgen activity is required for the dedifferentiation to occur.

Our K580R mutant is important in defining the functional interactions of AR with DNA and coactivators. Further characterization of this mutant will help understand the biology of AR.

**References:**

Marika J. Linja, Kimmo J. Savinainen, Outi R. Saramäki, Teuvo L.J. Tammela, Robert L. Vassella, and Tapio Visakorpi. (2001). Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. *Cancer Res.*, 61:3550-3555.

## Appendix I

**Molecular analysis of the androgen receptor in prostate cancer specimen obtained before and after hormonal treatment: androgen ablation selects an androgen receptor with expanded glutamine repeat.**

Dolores J. Lamb<sup>1,2</sup>, Efisio Puxeddu<sup>4,5</sup>, Rajni Nigam<sup>4,5</sup>, Nusrat Malik<sup>2</sup>, David Stenoien<sup>2</sup>, George Y. Saleh<sup>3,5</sup>, Micheal Mancini<sup>2</sup>, Nancy L. Weigel<sup>2</sup>, Marco Marcelli<sup>2,4,5,6</sup>. Scott Department of Urology<sup>1</sup>, Departments of Molecular and Cellular Biology<sup>2</sup>, Pathology<sup>3</sup>, and Medicine<sup>4</sup>. Baylor College of Medicine and VA Medical Center<sup>5</sup>, Houston TX, 77030.

Hormonal or androgen-ablative (AA) therapy is the predominant form of systemic treatment for metastatic prostate cancer. While an initial response to AA is observed in 70-80% of patients with advanced disease, most tumors eventually progress to androgen-independent growth and only a minority of affected individuals are alive 5 years following initiation of treatment. Since AA induces a dramatic change of the hormonal milieu of the patient and these tumors maintain the ability to proliferate, it is possible that this treatment selects a population of cells with a mutated androgen receptor, that sustain growth despite absence of circulating androgen. To test this hypothesis, we have investigated the molecular structure of AR in ten prostate cancer specimens obtained before and after AA. Tumors (coded A through L) were microdissected to extract genomic DNA uniquely from cells. Exons 1-8 of the androgen receptor were screened by PCR-SSCP and sequence analysis. A mutation, consisting in the expansion of the polyglutamine repeat from 20 (found in 100% of the sequences of the specimen obtained before AA) to 26 (found in 70% of the sequences of the specimen obtained after AA), was detected in patient F. ARQ26 readily translocated to the nucleus upon addition of androgen, and did not show significant differences in its ability to bind <sup>3</sup>H-DHT compared to its wild type counterpart. Nevertheless, analysis of transcriptional activity showed that the Q26 androgen receptor was 30-50% less active than the wild type molecule. Thus, it may be that cells carrying this partially inactive AR were less sensitive to the apoptotic effect of AA, and, unlike their counterparts carrying a WT AR, survived in the androgen-depleted environment of patient F.

## Appendix II

### Androgen receptor mutants identified in prostate cancer metastases that exhibit reduced activity.

N P Malik<sup>1\*</sup>, AJ James<sup>1</sup>, M Marcelli<sup>1,2</sup>, DJ Lamb<sup>1,3</sup>, and NL Weigel<sup>1</sup>. Molecular and <sup>1</sup>Cellular Biology, Baylor College of Medicine, Houston Tx 77030; <sup>2</sup>Medicine, Baylor College of Medicine, Houston Tx, 77030; and <sup>3</sup>Urology, Baylor College of Medicine, Houston Tx, 77030.

The role of androgen receptor mutations in prostate cancer has been a subject of much debate. Androgens stimulate the growth of prostate tumors and tumors from patients who have failed flutamide therapy sometimes express mutant androgen receptors that respond to flutamide as an agonist. In normal prostate, androgens induce expression of stromal cell growth factors that stimulate the growth of the epithelial cells. Androgen action in the epithelial cells induces synthesis of proteins characteristic of the differentiated state such as PSA. In the last few years, we have identified a number of androgen receptor mutations in lymph node metastases of prostate tumors from patients who have not previously undergone treatment. To date, our functional analyses show that the majority of the mutations that exhibit a phenotype, show reductions in either hormone dependent or ligand independent activity. Mutations have been detected both in the DNA binding domain and in the hormone binding domain. One previously described mutant C619Y, fails to bind to DNA and is transcriptionally inactive. Two other DNA binding domain mutants, A586V and A587S appear to show no change in transcriptional activity whereas a third, T575A shows enhanced activity. The other mutations are located in the hormone binding domain and exhibit a wide variety of phenotypes. Q919R responds reasonably well to ligand but has lost its capacity to be activated by forskolin, an activator of Protein Kinase A. V757A exhibits normal hormone binding, but its transcriptional activity is specifically reduced in prostate cancer cell lines. S863P does not bind hormone; it is transcriptionally inactive either in response to hormone or to forskolin. Preliminary data show that A748T also has reduced activity. These studies suggest that reduced androgen receptor activity early in the progression of prostate cancer may allow the epithelial cells to dedifferentiate and resume proliferation. Supported in part by NIH R01 CA68615 from the National Cancer Institute, CaP Cure, the Baylor SPORE on Prostate Cancer, DAMD17-99-1-9508 from the Department of Defence.





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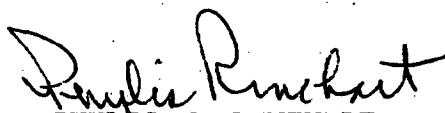
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